Dermal Fibroblasts Actively Metabolize Retinoic Acid but not Retinol

R. Keith Randolph* and Marcia Simon*†

*Living Skin Bank, University Hospital, Stony Brook, New York, U.S.A.; †Departments of Dermatology and Oral Biology and Pathology, School of Dental Medicine, SUNY at Stony Brook, Stony Brook, New York, U.S.A.

The accessibility of plasma retinol and retinoic acid to the epidermis may be influenced by the number and metabolic capacity of fibroblasts in papillary dermis. The metabolism of retinol-binding protein-bound all-*trans*-retinol, and albumin-bound all-*trans*-retinoic acid, by fibroblasts cultured on plastic dishes or in type I collagen gels, was examined. There were no significant differences in the metabolism of either retinoid by fibroblasts as a function of culture condition. There were large differences between retinoids, however. Retinoic acid was rapidly taken up and metabolized to unidentified polar metabolites that were released to the medium. Metabolic capacity was not saturated up to a medium retinoic acid concentration of 1 μM, and was induced further by prior exposure to retinoic acid. In contrast, retinol, although readily taken up, was not metabolized, i.e., neither retinoic acid nor retinyl ester was formed. By immunohistochemistry, the average number of fibroblasts in a 100 μm thickness of papillary dermis was estimated to be 1×10^6 cells per cm^2. Utilizing this value, the capacity of dermal fibroblasts to metabolize retinoic acid based on fibroblast abundance in the dermis was calculated. The results suggest that fibroblasts could limit delivery of plasma retinoic acid but not retinol to the epidermis on the basis of their metabolic capacity and abundance in the dermis. ***Key words: epidermis/high performance liquid chromatography/retinol-binding protein/skin/vitamin A. J Invest Dermatol 111:478–484, 1998***

Retinoic acid (RA) is an active form of vitamin A, retinol (Roh), that is essential for normal epithelial homeostasis (Gudas *et al*, 1994). This signaling molecule influences the transcription of retinoid-responsive genes in target cells by its reversible binding to nuclear retinoid receptors (reviewed by Gudas *et al*, 1994; Mangelsdorf *et al*, 1994). Two sources of RA are potentially available to epithelial tissues. First, RA can be generated in the skin following the uptake and local metabolism of plasma Roh that is transported bound to retinol-binding protein (Blaner and Olson, 1994). Second, in addition to Roh, RA is also present in the circulation bound to albumin (Lehman *et al*, 1972; Smith *et al*, 1973; for review, see Blaner and Olson, 1994) and is taken up by a variety of cell types, contributing significantly to tissue RA pools in some cases (Kurlandsky *et al*, 1995). Both Roh and RA are present in the plasma under normal physiologic circumstances, although at different concentrations. Retinol is present in human plasma bound to retinol-binding protein (RBP) at 1–2 μM (Blaner and Olson, 1994; Soprano and Blaner, 1994) and RA is present in human plasma at 4–14 nM (De LeenHeer *et al*, 1982; Eckhoff and Nau, 1990).

The differentiation program of epidermal keratinocytes is sensitive to exogenous retinoid, including Roh and RA (for reviews see Vahlquist and Törmä, 1988; Fuchs, 1990; Darmon, 1991). Normal differentiation is altered by excess or deficiency of either of these retinoids. For example, Asselineau *et al* (1989) have demonstrated that keratinocytes maintained on dermal equivalent cultures at the air–liquid interface exhibit a normal pattern of differentiation only when medium contained a suitably low concentration of serum. Two conclusions follow these and other similar observations. One, exogenous retinoid is essential for normal keratinocyte differentiation. Two, normal differentiation is observed when extracellular retinoid concentration falls within a critical range; concentrations either above or below this do not support normal differentiation.

The concentration of RA in intact, normal epidermis has been reported to be 20 nM or lower (Vahlquist, 1982; Kang *et al*, 1995; Duell *et al*, 1996), similar to that found in the plasma (De LeenHeer *et al*, 1982; Eckhoff and Nau, 1990). Despite the fact that this endogenous RA concentration must fall within the critical range required for sustaining epithelial homeostasis, neither its source(s) nor the mechanisms by which its levels are controlled are fully understood.

At least some RA in the epidermis may arise via synthesis from Roh. Cultured epidermal keratinocytes synthesize RA (Randolph and Simon, 1993, 1996; Kurlandsky *et al*, 1994), and although RA synthesis appears to be negatively feedback regulated, RA production persists despite the availability of exogenous RA (Randolph and Simon, 1996). This suggests that at least some RA synthesis in keratinocytes is constitutive. RA synthesis in keratinocytes appears to be controlled, at least in part, at the level of substrate Roh availability. This involves RA-mediated regulation of the Roh esterification reaction. Under RA-sufficient conditions, Roh is esterified, sequestering it from the enzymes of RA synthesis (Kurlandsky *et al*, 1996; Randolph and Simon, 1996). Excessive accumulation of RA is prevented not only by regulation of synthesis, but also by high capacity metabolism of RA in keratinocytes (Randolph and Simon, 1997). Thus, maintenance of an appropriate RA concentration in keratinocytes probably involves multiple homeostatic mechanisms operating in concert.

In addition to its synthesis *in situ* from Roh, RA may be available to the epidermis from the blood supply of underlying dermis. This possibility is an important consideration because the concentration of

Manuscript received February 24, 1998; revised April 21, 1998; accepted for publication May 13, 1998.

Reprint requests to: Dr. Marcia Simon, Living Skin Bank, SUNY at Stony Brook, Stony Brook, NY 11794.

Abbreviations: Roh, retinol; RBP, retinol-binding protein.

RA in the plasma and in the epidermis compare so closely, both at or below 20 nM. Moreover, cells in the dermis could alter plasma retinoids both qualitatively and quantitatively. The metabolism of RBP-bound Roh or albumin-bound RA by human fibroblasts, a major cell type of the dermis, has not been previously studied, and this work was undertaken to characterize the metabolism of RA and Roh by dermal fibroblasts under physiologic conditions inasmuch as possible *in vitro*. Metabolism studies were conducted on cells grown on plastic tissue culture dishes and within type I collagen gels in order to assess the impact of a dermis-like extracellular matrix.

The capacity of dermal fibroblasts to limit the availability of plasma retinoids to the epidermis is also dependent on the abundance of fibroblasts in papillary dermis. To assess this, the density of fibroblasts in human papillary dermis was estimated using immunohistochemistry.

MATERIALS AND METHODS

[11,12–^{3}H(N)]-all-*trans*-RA, and [11,12–^{3}H(N)]-all-*trans*-retinol, 50 Ci per mmol, was purchased from New England Nuclear (Boston, MA). All-*trans*-RA, all-*trans*-retinol, and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Ketoconazole was obtained from Research Diagnostics (Flanders, NJ). The authentic retinoid standards, 3,4-didehydro-all-*trans*-RA and 4-oxo-all-*trans*-RA, were the generous gift of Hoffmann-La Roche (Nutley, NJ).

Experimental protocol The overall experimental protocol for labeling and analyzing cell-associated retinoids has been described in detail previously (Randolph and Simon, 1993). Brief descriptions of the individual experimental elements are described below.

Cell culture Human dermal fibroblast strains were obtained from biopsies of three healthy adults. Stock cultures of cells were maintained in medium consisting of Dulbecco's minimum essential medium (Gibco BRL, Grand Island, NY), containing penicillin (1000 units per ml), streptomycin (1000 μg per ml), and 10% fetal bovine serum (Hyclone, Logan, UT). Stock cultures were plated at a density of 3.5×10^3 cells per mm^2 and were passaged at ≈75%–100% confluence, which was achieved within 5–7 d. For experiments, fibroblasts of the same strain and passage and from the same stock culture were grown in either 100 mm Falcon plastic dishes (Becton Dickinson Laboratories, Lincoln Park, NJ), or 25 mm Nunc tissue culture inserts (Marsh Biomedical Products, Rochester, NY), containing type I rat tail collagen (Upstate Biotechnology, Lake Placid, NY) suspended in 35 mm Falcon plastic plates. Cells were plated on plastic dishes as described above for stock cultures. Cells in collagen gels (2×10^5–1×10^6 cells per raft; 3 ml per gel) were suspended in the growth medium described above and also containing 2 mg type I rat tail collagen per ml and 1.6×10^{-3} N NaOH. Cultures of cells in the collagen gels and dishes were fed with 5 ml of the medium described above every other day. For the collagen gel cultures this volume of medium was distributed both above and below the gel such that the gel was completely and continuously submerged.

Fibroblasts for experiments were of passage six or less and were allowed to grow 7 or 8 d following plating (confluence for dish cultures) prior to initiation of experiments. During this growth interval, the collagen gels spontaneously contracted and detached from the tissue culture insert walls. The extent of contraction was generally 25%–50%. On the day of experiments, collagen gels were removed from the parent wells and transferred to new wells in order to eliminate cells that had migrated out of the collagen and had become attached to the plastic insert.

At the end of the experiments, medium was removed and was analyzed as previously described (Randolph and Simon, 1993). Fibroblasts in dishes were washed three times with phosphate-buffered saline and harvested by treatment with 0.025% trypsin. The resulting cell suspension was washed again in phosphate-buffered saline prior to analysis. Collagen gels were likewise washed three times with phosphate-buffered saline and were incubated with gentle agitation at 37°C for ≈30 min in a solution of collagenase (Boehringer, Indianapolis, IN) at a concentration of 3 mg per ml. The collagenase buffer consisted of 0.13 M NaCl, 0.01 M calcium acetate, and 0.02 M HEPES, pH 7.2. This treatment resulted in complete digestion of the collagen raft. The fibroblasts were then recovered by centrifugation followed by a phosphate-buffered saline wash as described above for dish cultures. The incubations with either trypsin or collagenase for dish or gel cultures, respectively, were of equal duration.

[^{3}H]Retinoid substrates All retinoids were stored in the dark under an atmosphere of N_2 at –20°C and when out of storage were kept on ice under amber fluorescent lighting (GE F40 Gold). [^{3}H]RA- or [^{3}H]Roh-containing experimental medium was prepared on the day of experiments. Both unlabeled and radiolabeled RA were purified by normal phase high performance liquid chromatography (HPLC; described below). Unlabeled and radiolabeled Roh were purified by reverse phase HPLC (described below).

On the day of the experiments, freshly purified [^{3}H]RA was dried under a stream of nitrogen and was then dissolved in 40 μl dimethylsulfoxide and added to a sterile 100 mg per ml solution of fatty acid-free BSA. The [^{3}H]RA-albumin preparation was added to basal medium containing 10% fetal bovine serum such that the final added albumin concentration was 1 mg per ml. Together the added BSA and the native albumin present in 10% fetal bovine serum yielded a final medium albumin concentration of ≈75 μM. This albumin concentration compares to an albumin concentration in dermal interstitial fluid of 300 μM (Rossing and Worm, 1981). The binding and stability of [^{3}H]RA in the BSA preparations and in experimental medium was evaluated as described previously (Randolph and Simon, 1997). Except as indicated, experiments were performed with a [^{3}H]RA concentration of ≈5 nM. In these studies, the albumin to RA mole ratio was ≈60,000, which compares with an albumin to RA mole ratio range of 43,000–150,000 in human plasma, based on a plasma RA concentration range of 4–14 nM (De LeenHeer *et al*, 1982; Eckhoff and Nau, 1990) and a plasma albumin concentration of 600 μM (Rossing and Worm, 1991). Ketoconazole was added to cultures dissolved in a dimethylsulfoxide vehicle. A 10 mM stock solution was added to cultures at a volume:volume ratio of 1000 to yield a final ketoconazole concentration of 10 μM.

Retinol-binding protein-[^{3}H]Roh complexes were prepared as described previously (Randolph and Ross, 1991), except that RBP was purified from fresh human plasma as described by Berni *et al* (1985).

Following preparation, the BSA-[^{3}H]RA, or the RBP-[^{3}H]Roh preparations were added to medium. For incubations with [^{3}H]RA, the medium consisted of the growth medium described above. For incubations with RBP-[^{3}H]Roh, medium consisted of the same growth medium, lacking serum, and containing 20 mg BSA per ml. All experimental medium was prepared on the day experiments were initiated. Experiments were initiated by the addition of medium containing radioisotopic-retinoid to cultures.

Retinoid analysis Following experimental incubations with [^{3}H]RA or RBP-[^{3}H]Roh, fibroblasts and medium were harvested separately, [^{3}H]retinoids were extracted and analyzed by HPLC. To 1 ml aliquots of a sonicated cell suspension or medium were added 50 pmol each of the following internal standards: 4-oxo-RA, 3,4-didehydroretinoic acid, and RA. Total [^{3}H]retinoids were extracted by the method described by Barua (1991). The organic solvents for this extraction all contained butylated hydroxytoluene (0.05%). The recovery of ^{3}H and internal standards by this extraction method was consistently greater than 96%. Extracts were dried under N_2 and dissolved in 100 μl of mobile phase, all of which was injected for HPLC analysis.

Two separate HPLC protocols were utilized during these studies. The first employed a reverse phase (Waters 3.9 mm × 300 mm C18 NovaPak analytical column) gradient system and was utilized for quantitation of the polar RA metabolites (Randolph and Simon, 1996, 1997). The second HPLC protocol, a normal phase system, was employed for the purification of all-*trans*-RA (Randolph and Simon, 1996). It consisted of a Waters NovaPak 3.9 × 150 mm silica analytical column and an isocratic mobile phase consisting of hexanes, dichloromethane, and glacial acetic acid (170:30:0.1; vol:vol:vol) with a flow rate of 1.5 ml per min.

The identity of all-*trans*-RA was checked by chromatography of the methylated derivative (Randolph and Simon, 1993) by reverse phase HPLC as described above.

Column effluent was monitored for ^{3}H by scintillation counting of 0.25 min fractions. Fractions for scintillation counting were collected directly into 7 ml scintillation vials, mixed with 3 ml Scintiverse BD (Fisher) and counted in an LKB Rackbeta scintillation counter. The efficiency of counting in this system was 35%.

Individual [^{3}H]labeled retinoids were identified by matching retention times of isotope peaks with those of the added internal standards. The individual [^{3}H]retinoids were quantitated by summing the background-corrected cpm in each peak. In some cases, background-corrected cpm were converted to pmol based on the specific activity of the original substrate [^{3}H]retinoid. Typical background radioactivity ranged between 25 and 40 cpm per fraction. Cell protein was determined by the method described by Markwell *et al* (1978).

Measurement of replicating cells Cells in S phase of the cell cycle were quantitated by staining cell suspensions of harvested cells with a bromodeoxyuridine (BrdU; Boehringer) kit. Prior to harvest, 8 d dish or gel cultures were incubated with growth medium containing 10 μM BrdU for 1 h at 37°C. Cultures were harvested as described above and cell suspensions were applied to slides for BrdU immunostaining. After BrdU staining, cell nuclei were counterstained with hematoxylin. A total of 500 nuclei were counted and the fraction of BrdU-stained nuclei was calculated.

Immunohistochemistry Human skin biopsies (breast skin from breast reduction surgery) were frozen at –20°C and were cryosectioned tangentially from the epidermal surface to a depth of ≈200 μm. Serial 5 μm tissue sections were fixed in acetone for 20 min at –20°C. Sections were then incubated with 10 μg per ml of a monoclonal antibody directed against fibroblast antigen (Ab-1; Oncogene Research, Cambridge, MA) for 1 h at 37°C in a buffer consisting of 0.05 M Tris, 0.9% NaCl, pH 7.4. This antibody has been previously characterized as specific for human dermal fibroblasts (Saalbach *et al*, 1996). The primary antibody was localized with Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) at a concentration of 10 μg per ml under the same conditions listed above for the primary antibody. Cell nuclei were stained with DAPI-containing Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Fibroblasts were counted in double exposure photographs where the Cy3 fibroblast and the DAPI nuclear stains colocalized. Using these criteria, the number of fibroblasts per cm^2 skin surface area was determined in a total of 100 μm of papillary dermis. Sections from three normal adult donors were counted.

RESULTS

The objective of the first series of experiments was to determine the capacity of dermal fibroblasts to metabolize exogenous RA. For this, the metabolites of fibroblasts grown on plastic or within type I collagen gels were examined by reverse-phase HPLC. Typical results are shown in the chromatograms depicted in **Fig 1**. As a control, experimental medium containing 5 nM [^{3}H]all-*trans*-RA was incubated under conditions identical to those for the cell incubations. This control medium was extracted and analyzed (**Fig 1*a***) to assess any decomposition of the RA substrate due to intrinsic instability or incubation conditions. Under these conditions the RA did not undergo appreciable decomposition. Normal phase HPLC analysis of the peak of tritiated-RA that is retained at ≈14.5 min revealed a mixture of ≈85% all-*trans*-RA, 15% 13-*cis*-RA, and a trace of 9-*cis*-RA (data not shown). These isomers of RA spontaneously formed upon addition of the all-*trans*-RA to the medium (this reverse-phase HPLC system does not resolve the stereoisomers of RA). The spontaneous isomerization of all-*trans*-RA attending its addition to cell culture medium has been observed previously (Randolph and Simon, 1997).

When extracts of fibroblasts were analyzed by reverse phase HPLC, only RA was evident (**Fig 1*b***). The quantity of RA in fibroblasts grown on plastic was approximately 2-fold greater than that in the fibroblasts in collagen gels. The apparent 4-fold difference seen in **Fig 1(*b*)** is due to a 2-fold difference in cell protein between the two cultures (see figure legend). Analysis of this RA peak by normal phase HPLC (data not shown) revealed the same mixture of all-*trans*- and 13-*cis*-isomers as that found in control medium mentioned above.

Analysis of medium from the fibroblast cultures demonstrated a peak of radioactivity eluting in the unretained volume of the column (Vo), as well as a peak corresponding to the parent RA (**Fig 1*c***). The polar metabolites eluting in the unretained volume of the column were not analyzed further. In the dish cultures approximately 2-fold more of the parent RA had undergone metabolism to the polar material that was unretained in this chromatography system. This does not represent a significant difference in metabolism between the two culture systems but rather reflects differences in cell protein (see figure legend).

In no instance were carbon-4 oxidized metabolites of RA observed. To control for recovery and detection of this retinoid metabolite, authentic 4-oxo-RA was added to the analyses as an internal standard and was recovered with high efficiency. In this HPLC system it exhibited a retention time of 5.5 min. Importantly, 3,4-didehydroretinoic acid was not detected in fibroblast cultures (retention time of 13.5 min). This is noteworthy because 3,4-didehydroretinoic acid is a metabolite of RA in cultured keratinocytes (Randolph and Simon, 1997).

The time course for uptake and metabolism of RA by cultured fibroblasts was examined (**Fig 2**). Fibroblasts rapidly took up medium RA; cellular concentrations increased ≈10-fold over that in medium by 3 h (**Fig 2*a***) and decreased steadily with further incubation. Concomitant with rapid RA uptake by cells was the disappearance of medium RA and the appearance of polar metabolites in cells (**Fig 2*a***) and the medium (**Fig 2*b***). By 6 h, 60% of medium RA had been metabolized. Metabolism of RA continued beyond 6 h but was much slower than that observed in the first 6 h of incubation. This was likely due to decreasing medium and cellular RA concentrations.

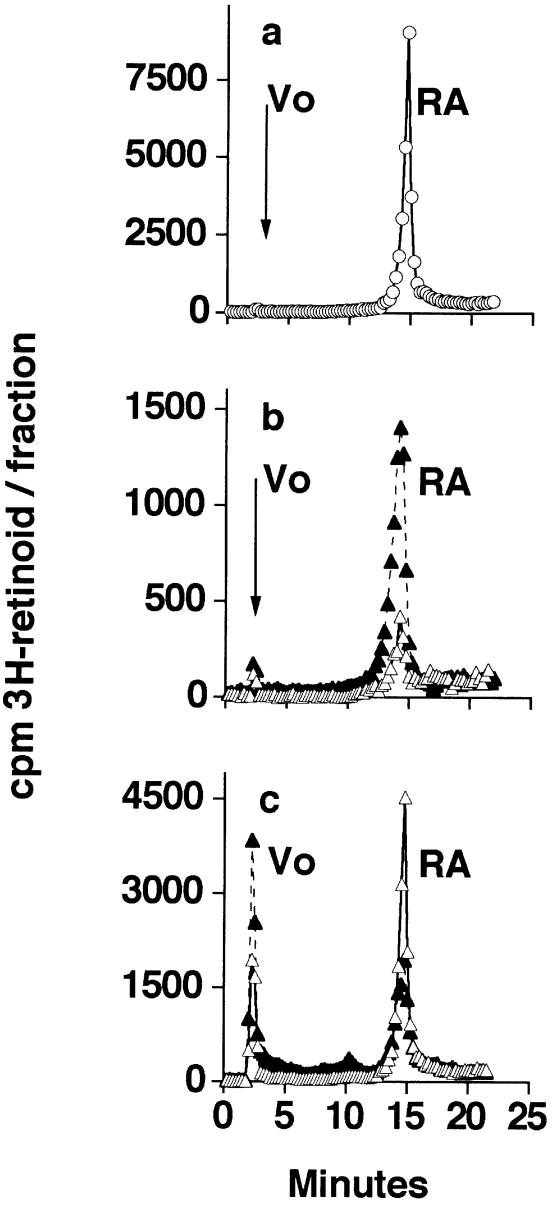

Figure 1. Fibroblasts grown on plastic or within collagen gels take up and metabolize exogenous RA similarly. Seven day old cultures of fibroblasts grown in plastic dishes or in collagen gels were incubated with medium containing 5 nM [^{3}H]RA for 24 h. Medium and cells were harvested separately and analyzed by reverse phase HPLC as described in *Materials and Methods*. The chromatogram in (*a*) represents the [^{3}H]RA recovered from control medium incubated for 24 h in the absence of cells. The chromatograms in (*b*) depict the radioactivity recovered from fibroblasts in collagen gel (*open symbols*) and dish (*closed symbols*) cultures. The chromatograms in (*c*) depict the radioactivity recovered from medium of the same fibroblasts cultures in collagen gels (*open symbols*) and dishes (*closed symbols*). Each chromatogram depicts typical results from all experiments and represents the average of three separate injections from triplicate incubations of dishes or gels. The retention time for authentic RA is 14.5 min. The unretained volume of the column (Vo) is indicated at 2.5 min. Approximately 1 mg of cell protein was analyzed from the dish cultures and ≈0.5 mg of cell protein was analyzed in the collagen gel cultures.

The uptake and metabolism of RA by fibroblasts were also determined as a function of medium RA concentration (**Fig 3**). The cellular concentration of RA increased linearly when medium RA was varied between 1 nM and 1 μM (**Fig 3*a***). Across the entire concentration

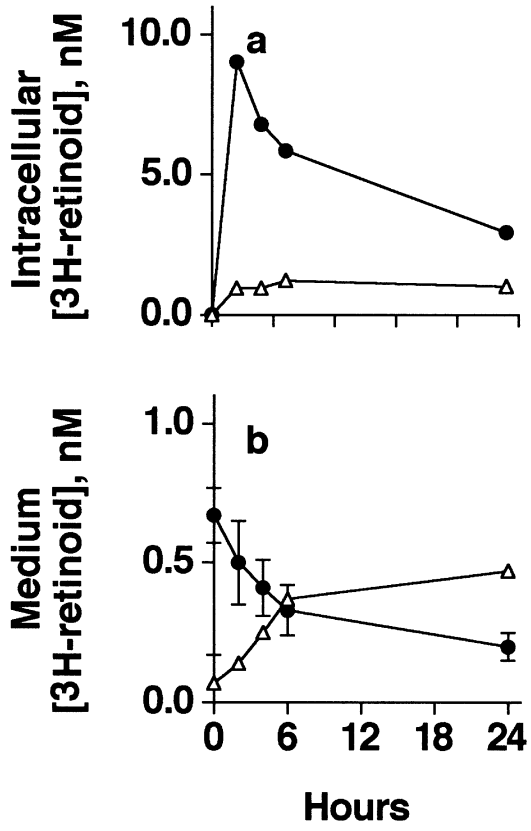

Figure 2. Exogenous RA is rapidly taken up and metabolized by cultured fibroblasts. Fibroblasts grown on plastic dishes were incubated with medium containing 0.7 nM [^{3}H]RA for the indicated times. Cells and medium were harvested separately and analyzed by HPLC. The concentrations of RA (*closed circles*) and its polar metabolites (radiolabeled retinoids eluting at Vo, *open triangles*) in cells (*a*) and medium (*b*) are shown. Values are the average of triplicate determinations from one experiment that was repeated with similar results. *Error bars*: ±SD.

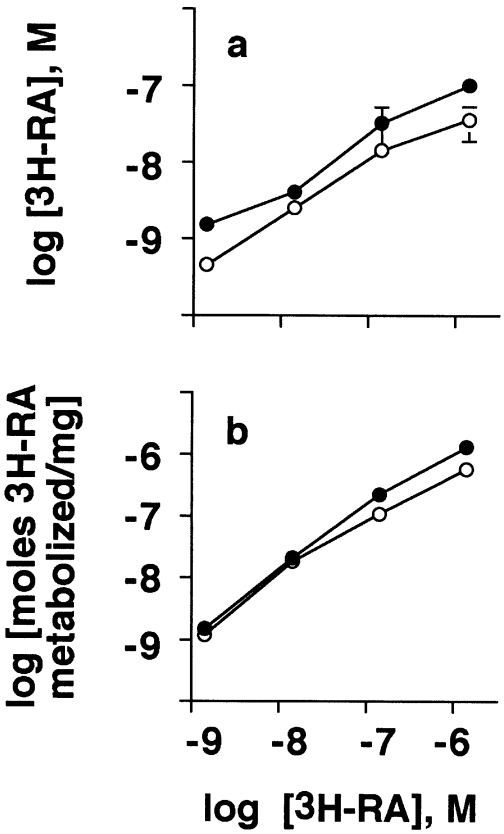

Figure 3. The uptake and metabolism of exogenous RA by cultured fibroblasts is not saturable at concentrations up to 1 μM. Fibroblasts cultured on plastic dishes (*closed symbols*) or collagen gels (*open symbols*) were incubated with medium containing the indicated concentrations of [^{3}H]RA for 24 h. Cells and medium were harvested separately and analyzed by HPLC. The molar concentration of RA in cells at 24 h is shown in (*a*). Data have been transformed by the log function. The total mole of polar metabolites produced per mg cell protein is shown in (*b*). Data have been transformed by the log function. The molar concentration of [^{3}H]RA in the original medium, shown on the x axis, has been log transformed. Values are the average of triplicate determinations from one experiment that was repeated with similar results. *Error bars*: ±SD.

range, cellular concentrations of RA were 2-fold higher in fibroblasts grown on plastic as compared with those grown within collagen gels.

Despite the difference in cellular RA concentrations between the two cultures, the rate of RA metabolism was approximately the same in the physiologic range (up to medium RA concentrations of 10 nM; **Fig 3*b***). Above this concentration RA metabolism occurred at a faster rate in fibroblasts cultured on plastic. Metabolism of RA was not saturable in either culture condition over this concentration range.

The metabolism of 5 nM [^{3}H]RA by fibroblasts grown on plastic and in collagen gels previously exposed to a pharmacologic concentration of RA was examined. Fibroblasts in both culture conditions increased the rate of RA metabolism by ≈50% as a result of pretreatment with 1 μM RA (data not shown).

The two culture systems were next compared for their sensitivity to ketoconazole, an inhibitor of cytochrome P450-linked RA metabolism. Incubation of cells in both culture systems with 10 μM ketoconazole inhibited RA metabolism by 50%–75% (data not shown).

By day 8 of culture, when experiments were performed, the cell protein recovered from the raft cultures was approximately half that recovered from the dish cultures (0.5 mg protein per gel *versus* 1 mg protein per dish). This suggested that fibroblasts cultured in collagen gels grew more slowly than fibroblasts cultured on plastic. This was an encouraging observation because a very low proportion of dermal fibroblasts *in vivo* are replicating (Kaye *et al*, 1971; Sarber *et al*, 1981; Van Neste and Lachapelle, 1981). To discern if the very high rate of RA metabolism was related to the percentage of dividing cells, BrdU incorporation was determined. On day 8 of culture, the percentage of BrdU positive cells in gels was 1%, and on plastic it was 12%.

In addition to albumin-bound RA, the circulatory system of the skin delivers RBP-Roh to fibroblasts of papillary dermis. Toward assessing the metabolic fate of this abundant retinoid substrate in the dermis, medium containing 1 μM RBP-bound [^{3}H]Roh was incubated with fibroblasts cultured within collagen gels or in plastic dishes for 24 h (isotopic steady state), and the tritium-labeled retinoid metabolites were extracted and analyzed by HPLC (**Fig 4**). The two radiochromatograms in **Fig 4(*a*)** are representative of extracts of fibroblasts in the two culture systems. Retinol was taken up similarly by cells in both culture systems and was present at an intracellular concentration of ≈2 μM. Strikingly, and despite the micromolar intracellular concentration, there were no metabolites of Roh detected within cells cultured in either condition, including RA (retention time of 15 min) or retinyl esters (retention times of 25–35 min) that could be attributed to metabolic activity of the cells. The trace amounts (<50 nM) of polar metabolites (Vo) and RA that were detected (not evident in the chromatogram due to scale of y axis), were present at similar concentrations in control medium incubated in the absence of cells (data not shown). Analysis of medium from the same cultures (**Fig 4*b***) likewise resulted in the recovery of essentially only the parent Roh.

Consistent with these radiolabeled studies in other experiments, the endogenous retinoids present in mass cultured fibroblasts (cells from ten 180 cm^2 flasks) grown in medium containing 10% fetal bovine serum (≈50 nM RBP-Roh) were analyzed. In these experiments, by optical detection (photodiode array detection), the only retinoid detected was Roh (data not shown).

In order to quantitatively predict the metabolic potential of fibroblasts

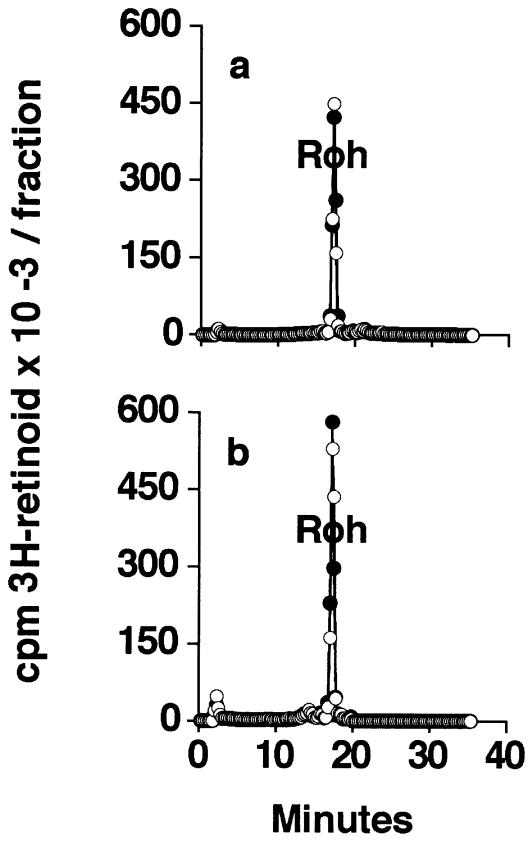

Figure 4. Cultured fibroblasts do not metabolize RBP-Roh. Fibroblasts cultured on plastic dishes (*closed symbols*) or within collagen gels (*open symbols*) were incubated with medium containing the 1 μM RBP-[^{3}H]Roh for 24 h (isotopic steady state). Cells (*a*) and medium (*b*) were harvested separately and analyzed by HPLC. Approximately 2 mg cell protein were analyzed in each culture condition. Radiochromatograms represent the average of triplicate samples. The experiment was repeated with similar results.

in vivo, it was necessary to determine the abundance of fibroblasts in papillary dermis. To accomplish this, fibroblasts were identified immunohistochemically in serial, tangential sections of papillary dermis (**Fig 5**) using an excellent monoclonal antibody specific for human dermal fibroblasts (Saalbach *et al*, 1996). The photomicrograph in **Fig 5**(***a***) is a low power view of a typical section and serves to illustrate the overall tissue distribution of fibroblasts when viewed tangentially to the skin surface. Fibroblasts in papillary dermis and in individual dermal papillae were very close to the epidermis, in many instances less than 5–10 μm away. Moreover, dense clusters of fibroblasts completely covered the extravascular side of capillaries. Also noteworthy were the striking long, thin, spindle-shaped processes of papillary fibroblasts (note the dashed box in **Fig 5***a*) that are not so evident in traditional cross-sections. The photomicrograph in **Fig 5**(***b***) shows an enlargement of the cells in the dashed box from **Fig 5**(***a***). The slender fibroblast processes were up to 100 μm in length, and tortuous, and formed a sieve-like network that completely filled the papillary dermis.

The number of fibroblasts per unit volume of papillary dermis was determined by counting fibroblasts in photomicrographs where the fibroblast-specific (red) and the nuclear (blue) stains localized to the same cell. In the immediate vicinity of blood vessels and hair follicles, the density of fibroblasts was so high that it was impossible to discern individual cells. In such cases we did not attempt to score cells. The data on fibroblast numbers thus represent a lower limit of dermal fibroblast density. Fibroblasts were counted in photomicrographs from sections representing a total of 100 μm thickness of papillary dermis. A typical photomicrograph that was scored for fibroblast numbers is shown in **Fig 5**(***c***). Sections from biopsies of three normal individuals were scored. The average fibroblast abundance in papillary dermis from these biopsies was 1×10^6 cells per cm^2 skin surface area in 100 μm of dermal tissue (SD; 0.28×10^6 per cm^2).

DISCUSSION

These studies have examined the uptake and metabolism of RA and Roh delivered in physiologic form and concentration by fibroblasts. Under these conditions, fibroblasts actively metabolize RA but not Roh. In a recent publication by Bailly *et al* (1998), the metabolism of pharmacologic RA and Roh by human dermal fibroblasts was reported. In their studies, micromolar RA or Roh was delivered to cultured human dermal fibroblasts in dispersed form, dissolved in ethanol. Under these conditions, fibroblasts exhibit a robust metabolism of RA to polar metabolites, similar to the present results. In contrast to the present studies, however, cultured human fibroblasts appear to convert solvent-dispersed Roh into trace amounts of RA and modest quantities of Roh ester (Bailly *et al*, 1998). The administration of Roh to cells in this manner delivers up to 10-fold more Roh to cells as compared with that which occurs when Roh is delivered in physiologic bound form, bound to RBP (Hodam *et al*, 1991). It seems likely that these differences are due to the availability of a very high substrate Roh concentration to low affinity pathways of metabolism that might be operative only under pharmacologic conditions. In addition, caution must be taken in interpreting the presence of RA in cells or medium when Roh is added to cells in solvent-dispersed form. This manner of adding Roh to tissue culture medium can result in some spontaneous oxidation of Roh into RA and other polar metabolites (Randolph and Simon, 1993).

Cultured fibroblasts exhibit a high capacity to metabolize RA presented in physiologic form bound to albumin. Does the metabolic capacity of fibroblasts have potential to play a quantitatively significant role in limiting the availability of plasma RA to the skin?

The quantitative significance of RA metabolism in fibroblasts can be gauged by evaluating their metabolic capacity in the context of extracellular RA concentrations likely to be encountered *in vivo*. Although the concentration of RA in human dermis is not known, it is possible to predict the highest concentration to which dermal fibroblasts might be exposed. The concentration of albumin in skin blister fluid has been reported to be around 300 μM, approximately half its concentration in plasma (Rossing and Worm, 1981). If plasma albumin permeating into the dermal interstitium retains its function as the physiologic transport protein for RA in the plasma (Lehman *et al*, 1972; Smith *et al*, 1973), the concentration of RA in the dermis (derived from plasma) should be about one-half of its concentration in plasma (De LeenHeer *et al*, 1982; Eckhoff and Nau, 1990), or ≈5 nM. This represents a conservative estimate of RA concentration because it assumes that there is no metabolism or synthesis by other cell types. Dermal fibroblasts clearly possess ample biochemical capacity to metabolize exogenous RA at this concentration (**Fig 3**). In fact, RA metabolism in fibroblasts is not saturated at RA concentrations 100-fold higher than those expected *in vivo*. Thus, if fibroblasts express a similarly robust capacity for RA metabolism *in vivo*, metabolism would be limited by factors other than biochemical potential.

The dermis is rich in extracellular matrix and, from a histologic point of view, is less cellular than the overlying epidermis. Is RA metabolism by fibroblasts quantitatively significant on the basis of cellular abundance?

The number of fibroblasts per unit volume of a 100 μm thickness of papillary dermis has been estimated to be 1×10^6 fibroblasts per cm^2 skin surface area. Assuming an extracellular RA concentration of 5 nM (see above), fibroblasts metabolize 5 pmol RA per 10^6 cells per day (**Fig 3**). Multiplying this value times the number of fibroblasts per unit volume of dermis, 1×10^6 cells per cm^2, gives a tissue fibroblast metabolic capacity of 5 pmol RA per cm^2 per day, assuming a constant extracellular RA concentration of 5 nM.

The importance of this metabolic potential must be evaluated in the context of the exogenous RA supply. The extracellular supply of RA depends on the RA concentration of interstitial fluid and its rate of flow through the dermis, a value that can be estimated. The RA concentration in dermal interstitial fluid is estimated to be ≈5 nM (see

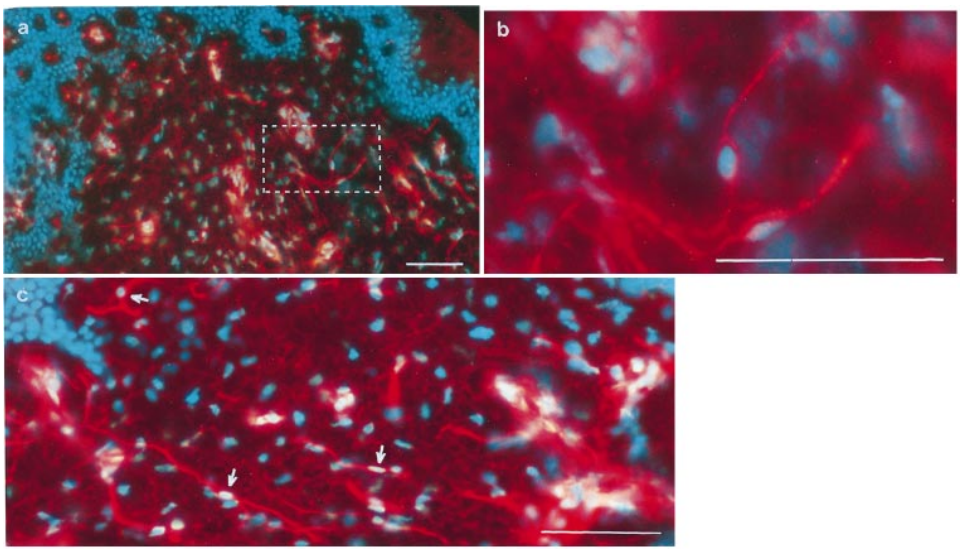

Figure 5. Immunohistochemistry of fibroblasts in tangential sections of human dermis. A biopsy of human skin was frozen at –20°C and 5 μm tangential sections of papillary dermis were prepared and stained as described in *Materials and Methods* for a fibroblast-specific antigen. Red staining depicts localization of the fibroblast-specific antibody. All cell nuclei are stained blue. (*a*) A low power view (10×) of a typical section through the papillary dermis. The "band" of blue nuclear staining (basal keratinocyte nuclei) across the top of the photograph depicts where the section skimmed the overlying epidermis (above the plane of the photograph). Individual dermal papillae can be seen as the "holes" in the band of blue nuclei. Note the red staining within the core of each papillae. The dashed inset depicts the region of this field that was photographed at high power (40×), shown in (*b*). (*b*) A high power view (40×) of fibroblasts exhibiting typical morphology for papillary dermis. (*c*) A typical photomicrograph (20×) that was used to score the numbers of fibroblasts in dermis. Fibroblasts were counted in photomicrographs using the criteria of colocalization of nuclear (blue) and fibroblast (red) staining. Some examples of this colocalization are indicated with the white arrows. *Scale bars*: 100 μm.

above). Because the concentration of albumin in skin blister fluid (Rossing and Worm, 1981), interstitial fluid, and lymph (Reed *et al*, 1993) are similar, the flux of plasma RA (albumin-bound RA) through dermal interstitium is assumed to approximate the lymph flow from the skin (Reed *et al*, 1993). The lymph flow from the skin is proportional to the proportion of cardiac output to the skin, ≈5% (Tan and Stafford, 1987). Total lymph flow in an adult is ≈5 liters per day (Berne and Levy, 1997). Adult body surface area is assumed to be 2 m^2. The estimated supply of RA (originating from the plasma) to dermis in 1 cm^2 of skin is as follows: 5 liters lymph per day × 0.05 × 5 nM RA/liter ÷ 20,000 cm^2 = 0.06 pmol RA per cm^2 per day.

This calculated supply of plasma RA to the dermis is strikingly less than the metabolic capacity of fibroblasts in a 100 μm thickness of dermal tissue (5 pM per cm^2 per day). Thus, the capacity of RA metabolism in dermal fibroblasts exceeds the predicted extracellular supply of RA from plasma both on a biochemical and on a cellular level.

Is RA metabolism in the dermis or epidermis quantitatively important in maintaining low concentrations of RA in the epidermis? Insight to this question can be gained by comparing the RA content of the epidermis with the estimated supply of RA from the microvasculature of underlying dermis. In the discussion above, assuming no additional input from synthesis (**Fig 4**), and no output via metabolism, the estimated supply of plasma RA to the epidermis is 0.06 pmol RA per cm^2 of skin surface area per day. If this quantity of RA was retained in a 100 μm thickness of epidermis of the same surface area, its concentration would increase by 6 pmol per cm^3 (6 nM) per day. This increment is in the range of the RA concentration of epidermis (<20 nM; Kang *et al*, 1995; Duell *et al*, 1996). Under the limitations of these assumptions, substantial RA metabolism must occur in the vicinity of the dermal–epidermal interface. These data suggest that this could occur, at least in part, in fibroblasts of papillary dermis.

Recent results from this laboratory have demonstrated that cultured keratinocytes also exhibit high capacity to metabolize exogenous RA (Randolph and Simon, 1997). Cultured keratinocytes resemble the keratinocytes that are present in wound healing epithelium (Mansbridge and Knapp, 1987). Replicating keratinocytes are located in the basal layer of the epidermis, immediately above the papillary dermis and beneath the differentiating suprabasal cells. Fibroblasts of the papillary dermis are distributed around the vascular supply of the dermis and immediately beneath the epidermis. These two cell populations are in close proximity to each other and could function together as a metabolic barrier, limiting the access of extracellular RA to differentiating suprabasal cells of the epidermis.

Epidermal cells are known to require low concentrations of active retinoid to maintain normal differentiation (Asselineau *et al*, 1989). If plasma RA from the dermis has limited availability to the differentiating epidermal cells due to metabolism at the dermal–epidermal interface, other sources of RA must be considered for sustaining epidermal homeostasis. One such source is the synthesis of RA from Roh. Retinol is clearly available to the epidermis *in vivo* as significant quantities of Roh and Roh esters are present in intact epidermis (Vahlquist and Törmä, 1982). This study suggests that RBP-Roh permeating into dermal interstitium from the blood supply would not be significantly metabolized by dermal fibroblasts en route to the epidermis. This Roh would thus be available to epidermal cells for the synthesis of RA. In line with this possibility, a number of laboratories have reported the synthesis of RA from Roh in intact keratinocytes and in keratinocyte extracts (Siegenthaler *et al*, 1990; Randolph and Simon, 1993, 1996; Kurlandsky *et al*, 1994, 1996; Chatellard-Gruaz *et al*, 1998). Thus, suitable concentrations of RA in the epidermis could be maintained by a balance between limited RA synthesis *in situ* and active RA metabolism.

This work was funded in part by Unilever Research U.S.

REFERENCES

Asselineau D, Bernard BA, Baiklly C, Darmon M: Retinoic acid improves epidermal morphogenesis. *Dev Biol* 133:322–335, 1989

Bailly J, Crettaz M, Schifflers MH, Marty JP: In vitro metabolism by human skin and fibroblasts of retinol, retinal and retinoic acid. *Exp Derm* 7:27–34, 1998

Barua AB: Analysis of water-soluble compounds. Glucuronides. *Meth Enzymol* 189:136–145, 1991

Berne RM, Levy MN: The microcirculation and lymphatics. In: Berne RM, Levy MN (eds). *Cardiovascular Physiology*, 7th edn. St Louis: CV Mosby, 1997, pp. 153–170

Berni R, Ottonello S, Monaco HL: Purification of human plasma retinol-binding protein by hydrophobic interaction chromatography. *Anal Biochem* 150:273–277, 1985

Blaner WS, Olson JA: Retinol and retinoic acid metabolism. In: Sporn MB, Roberts AB, Goodman DS (eds). *The Retinoids: Biology, Chemistry, and Medicine*. 2nd edn. New York: Raven Press, 1994, pp. 283–318

Chatellard-Gruaz D, Randolph RK, Hagens G, Saurat JH, Siegenthaler G: Differentiation of human epidermal keratinocytes is accompanied by increased expression of CRABP-II and increased cellular concentration of retinoic acids: Retention of newly synthesized retinoic acids by CRABP-II. *J Lipid Res* in press, 1998

Darmon M: Retinoic acid in skin and epithelia. *Sem Dev Biol* 2:219–228, 1991

De LeenHeer AP, Lambert WE, Claeys I: All-*trans*-retinoic acid: Measurement of reference values in human serum by high performance liquid chromatography. *J Lipid Res* 23:1362–1367, 1982

Duell EA, Kang S, Voorhees JJ: Retinoic acid isomers applied to human skin *in vivo* each induce a 4-hydroxylase that inactivates only *trans* retinoic acid. *J Invest Dermatol* 106:316–320, 1996

Eckhoff C, Nau H: Identification and quantitation of all-*trans*- and 13-*cis*-retinoic acid and 13-*cis*-4-oxo-retinoic acid in human plasma. *J Lipid Res* 31:1445–1454, 1990

Fuchs E: Epidermal keratinization: The bare essentials. *J Cell Biol* 111:2807–2814, 1990

Gudas LJ, Sporn MB, Roberts AB: Cellular biology and biochemistry of the retinoids. In: Sporn MB, Roberts AB, Goodman DS (eds). *The Retinoids: Biology, Chemistry, and Medicine*. 2nd edn. New York: Raven Press, 1994, pp. 443–520

Hodam JR, St Hilaire P, Creek KE: Comparison of the rate of uptake and biological effects of retinol added to human keratinocytes either directly to the culture medium or bound to serum retinol-binding protein. *J Invest Derm* 97:298–304, 1991

Kang S, Duell EA, Fisher GJ, *et al*: Application of retinol to human skin *in vivo* induces epidermal hyperplasia and cellular retinoid binding proteins characteristic of retinoic acid but without measurable retinoic acid levels or irritation. *J Invest Dermatol* 105:549–556, 1995

Katelaris A, Penny R, Breit SN: Mitotic activity in the dermis of mice during graft versus host disease: The role of fibroblast replication in dermal fibrosis. *J Invest Dermatol* 99:779–783, 1992

Kaye GI, Siegel LF, Pascal RR: Cell replication of mesenchymal elements in adult tissues I. The replication and migration of mesenchymal cells in the adult rabbit dermis. *Anat Rec* 169:593–612, 1971

Kurlandsky SB, Xiao J, Duell EA, Voorhees JJ, Fisher GJ: Biological activity of all-*trans*-retinol requires metabolic conversion to all-*trans*-retinoic acid and is mediated through activation of nuclear retinoid receptors in human keratinocytes. *J Biol Chem* 269:32821–32827, 1994

Kurlandsky SB, Gamble MV, Rajesekhar R, Blaner WS: Plasma delivery of retinoic acid to tissues in the rat. *J Biol Chem* 270:17850–17857, 1995

Kurlandsky SB, Duell EA, Kang S, Voorhees JJ, Fisher GJ: Auto-regulation of retinoic acid biosynthesis through regulation of retinol esterification in human keratinocytes. *J Biol Chem* 271:15346–15352, 1996

Lehman ED, Spivey HO, Thayer RH, Nelson EC: The binding of retinoic acid to serum albumin in plasma. *Fed Proc* 31:A672, 1972

Mangelsdorf DJ, Umesono K, Evans RM: The retinoid receptors. In: Sporn MB, Roberts AB, Goodman DS (eds). *The Retinoids: Biology, Chemistry, and Medicine*. 2nd edn. New York: Raven Press, 1994, pp. 319–350

Mansbridge JN, Knapp AM: Changes in keratinocyte maturation during woulnd healing. *J Invest Dermatol* 89:253–263, 1987

Markwell MAK, Haas SM, Bieber LL, Tolbert NE: A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 87:206–210, 1978

Randolph RK, Ross AC: Regulation of retinol uptake and esterification in MCF-7 and HepG2 cells by exogenous fatty acids. *J Lipid Res* 32:809–820, 1991

Randolph RK, Simon M: Characterization of retinol metabolism in cultured human epidermal keratinocytes. *J Biol Chem* 268:9198–9205, 1993

Randolph RK, Simon M: all-*trans*-retinoic acid regulates retinol and 3,4-didehydroretinol metabolism in cultured human epidermal keratinocytes. *J Invest Dermatol* 106:168–175, 1996

Randolph RK, Simon M: Metabolism of all-*trans*-retinoic acid by cultured human epidermal keratinocytes. *J Lipid Res* 38:1374–1383, 1997

Reed RK, Ishibashi M, Townsley MI, Parker JC, Taylor AE: Blood-to-tissue clearance vs. lymph analysis in determining capillary transport characteristics for albumin in skin. *Am J Physiol* 264:H1394–H1401, 1993

Rossing N, Worm AM: Interstitial fluid: Exchange of macromolecules between plasma and skin interstitium. *Clin Phys* 1:275–284, 1981

Saalbach A, Anderegg U, Bruns M, Schnabel E, Herrmann K, Haustein UF: Novel fibroblast-specific monoclonal antibodies: properties and specificities. *J Invest Dermatol* 106:1314–1319, 1996

Sarber R, Hull B, Merrill C, Soranno T, Bell E: Regulation of proliferation of fibroblasts of low and high population doubling levels grown in collagen lattices. *Mech Ageing Dev* 17:107–117, 1981

Siegenthaler G, Saurat JH, Ponec M: Retinol and retinal metabolism. Relation to the state of differentiation of cultured human keratinocytes. *Biochem J* 268:371–378, 1990

Smith JE, Milch PO, Muto Y, Goodman DS: The plasma trasport and metabolism of retinoic acid in the rat. *Biochem J* 132:821–827, 1973

Soprano D, Blaner WS: Plasma retinol-binding protein. In: Sporn MB, Roberts AB, Goodman DS (eds). *The Retinoids: Biology, Chemistry, and Medicine*. 2nd edn. New York: Raven Press, 1994, pp. 257–282

Tan OT, Stafford TJ: Cutaneous circulation. In: Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF (eds). *Dermatology in General Medicine*. 3rd edn. New York: McGraw-Hill, 1987, pp. 357–367

Vahlquist A: Vitamin A in human skin: I. Detection and identification of retinoids in normal epidermis. *J Invest Dermatol* 79:89–93, 1982

Vahlquist A, Törmä H: Retinoids and keratinization. *Int J Derm* 27:81–95, 1988

Van Neste D, Lachapelle JM: A comparative study of patterns of [^{3}H]thymidine labeling in epidermal and dermal cells of allergic contact dermatitis, irritant dermatitis and atopic dermatitis. In: Marks R, Christophers E (eds). *The Epidermis in Disease*. Philadelphia: JB Lippincott, 1981, pp. 211–222